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ORIGINAL PAPER

Investigations on inulin-type oligosaccharides with regard to HPLC analysis and prospective food applicability

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Abstract Relevance of research on prebiotic food components is recently largely enhanced as consumption of health-promoting functional foodstuffs displays a definite increase. Since there is a direct correlation between oligomer distribution of inulin and its prebiotic effect, it is of crucial importance to be capable to characterize its actual composition in thermally treated food samples. A newly developed high performance liquid chromatography method is applied for the examination of plant inulin samples using evaporative light scattering detection. The procedure contains a direct detection procedure of native inulin/fructo-oligosaccharide components after dissolving the samples in water at room temperature and by detecting the chain-distribution with HPLC coupled with evaporative light scattering detection. The method is accurate, simple, and without interferences from the detectable signals of mono- and disaccharides as thermal decomposition products of inulin. The analytical procedure eliminates the need to use artificial chemical hydrolysis of the macromolecule. The fructo-oligosaccharide components have been investigated by mass spectrometric detection with APCI ionization. The composition of several industrial samples and the thermal properties of inulin have also been investigated in order to reveal exact composition of inulin comprising bakery products exposed to thermal treatment. The thermal degradation leads to increase of the prebiotic feature as well.

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Introduction

Inulin-type fructans [1] (fructo-oligosaccharides) belong to a naturally occurring oligosaccharide family produced by several thousand plant species (fruits and vegetables) [2], therefore they might be regarded as very frequently occurring carbohydrate derivatives in nature [3]. Natural inulin is a polydisperse mixture of carbohydrate oligomers with mostly linear (or branched in few percentage [4]) chains containing fructose units (connected through exclusively β-(1 → 2) fructosyl–fructose linkages) having a terminal glucose or fructose moiety. In higher plant fructo-oligosaccharides (stored as reserve carbohydrates) the number of monomers (degree of polymerization, DP) does not exceed 200. The number and the distribution of different oligomers show characteristic indication of the inulin producing plant.

Inulins with a terminal glucose unit are known as α-D-glucopyranosyl-[β-D-fructofuranosyl](n-1)-D-fructofuranosides (or fructo-oligosaccharides), which are abbreviated GF_n, while derivatives without terminal glucose belong to the β-D-fructopyranosyl-[D-fructofuranosyl](n-1)-D-fructofuranosides (or inulo-oligosaccharides) group, abbreviated as F_n, where n refers to the number of fructose residues (structural formulas are shown in Fig. 1).

As a result of the β configuration at the anomeric carbon atom of the fructose monomers, inulin-type fructans resist the hydrolysis by human intestinal, small digestive enzymes and therefore they might be considered as

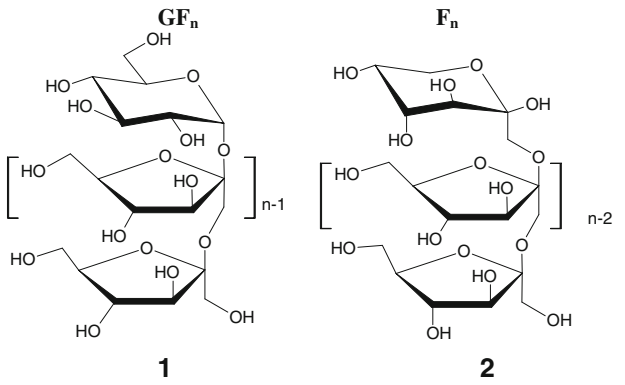

Fig. 1 The general structure of inulin type linear fructo-oligosaccharides: GF_n type oligomer with n fructose units in a chain terminated with a glucose unit (**1**) and F_n type oligomer constructed exclusively by fructose units (**2**)

non-digestible oligosaccharides (NDOs) [5–7]. These oligosaccharides have important physiological properties, prebiotic activity [8, 9], and act as dietary fibers having beneficial effect on the intestinal bacterial populations, biochemical, physiological, and physicochemical [10] processes. As a result of the high biological activity, intensity of food industrial driven research on such type of compounds aiming at prospective practical applications has increased in the last years [11, 12].

Several human physiologically beneficial properties have been associated with the non-digestible oligosaccharides, such as the favorable modification of the colon microflora, pH decrease in the colon, enhanced nutrient production (e.g. B vitamins), increased mineral absorption (iron, calcium, magnesium), beneficial effect on the lipid and carbohydrate metabolism, and cancer risk reduction [5–8, 11, 13–20].

Inulin type fructans are water soluble, however, their sweetness is approximately one tenth of sucrose, moreover the sweetness depends on the structure, the degree of polymerization, and the ratio of the different derivatives existing in the mixture [12, 20]. Furthermore, the sweetness has been found to follow a decreasing tendency with increasing chain length [5]. The low sweetness has initiated many food industrial applications to imply inulin-type fructans as diabetic food components [11, 21].

Thermal treatment of inulin-type oligosaccharides results in chain degradation, monomers and short chain length derivatives appear in the mixture of the decomposition products [21–23]. Interestingly, thermal treatment significantly stimulates the growth of bifidobacteria and suppresses the growth of possibly pathogenic bacteria [24]. This characteristic feature underlines the significance and great perspectives of possible industrial implications by utilization of inulin as functional food component.

Since inulin-type oligosaccharides show highly heterogeneous distribution of different oligomers, qualitative and quantitative analytical characterization is a challenging task. Inulin oligomers have been analyzed by HPLC methods using different detection techniques, however UV/Vis detection has provided us with poor results in terms of sensitivity, mainly due to the weak UV-absorbing properties of native carbohydrate derivatives. High-performance anion exchange chromatography with pulsed amperometric detection (HPAE–PAD) has been applied to the analysis of inulin samples [25] along with refractive index (RI) detection methods [26, 27]. However, these methods require special chromatographic arrangements, PAD and RI detections are sensitive to eluents and the applied gradient elution.

Our intention was to develop a reliable HPLC method for proper qualitative and quantitative analysis of inulin oligomers and degradation products in order to provide a correct estimation of the composition of thermally treated food products, as well as lay the basis of assessment of the prebiotic effect of thermally treated inulin containing food samples. The HPLC method was set up using a carbohydrate column ($–NH_2$ funtionalities on the stationary phase) and evaporative light scattering (ELS) detection. Inulin powder samples of *Chicorium intybus L.* (chicory) and *Helianthus tuberosus* (Jerusalem artichoke) were studied along with industrial food samples. Thermal treatments were carried out at two elevated temperatures (180 and 210 °C) in order to characterize diversely the thermal degradation process of inulin and determine all the produced oligo- and polymers. The identification of different oligomer components was carried out in HPLC–MS (APCI) experiments.

Results and discussion

The retention times of the three standard signals (1-kestose, 1,1-kestotetraose and 1,1,1-kestopentaose, see Fig. 2 for structures) have been determined at 10.96, 13.06, and 15.15 min (Fig. 3) by applying the HPLC method coupled with evaporative light scattering detection. The separation was good enough to apply the method for inulin samples containing oligomers with higher polymerization degree. The signals in the HPLC chromatogram (Fig. 3) of the standard inulin sample appeared within an 80 min time interval with detectable components between DP3 and DP36 in an almost equidistant appearance. The highest signal intensity was found to be attributed to DP4, the intensity of higher molecular weight oligomers showed exponential decrease with increasing retention time.

The chromatogram of inulin sample containing oligomers with low polymerization degree was acquired also

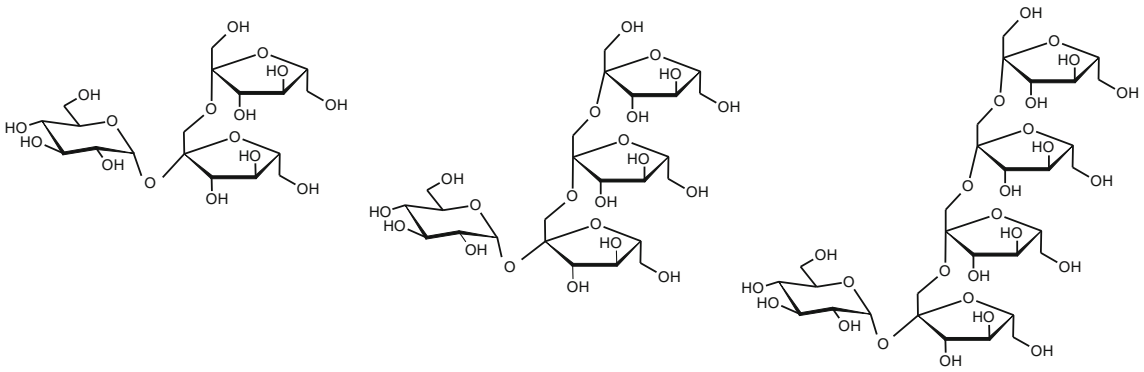

Fig. 2 The structure of the first three oligomers used as standards in HPLC experiments: 1-kestotriose (1-kestose—DP3), β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside (**3**); 1,1-kestotetraose (nystose—DP4), β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside (**4**); and 1,1,1-kestopentaose (DP5), β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside (**5**)

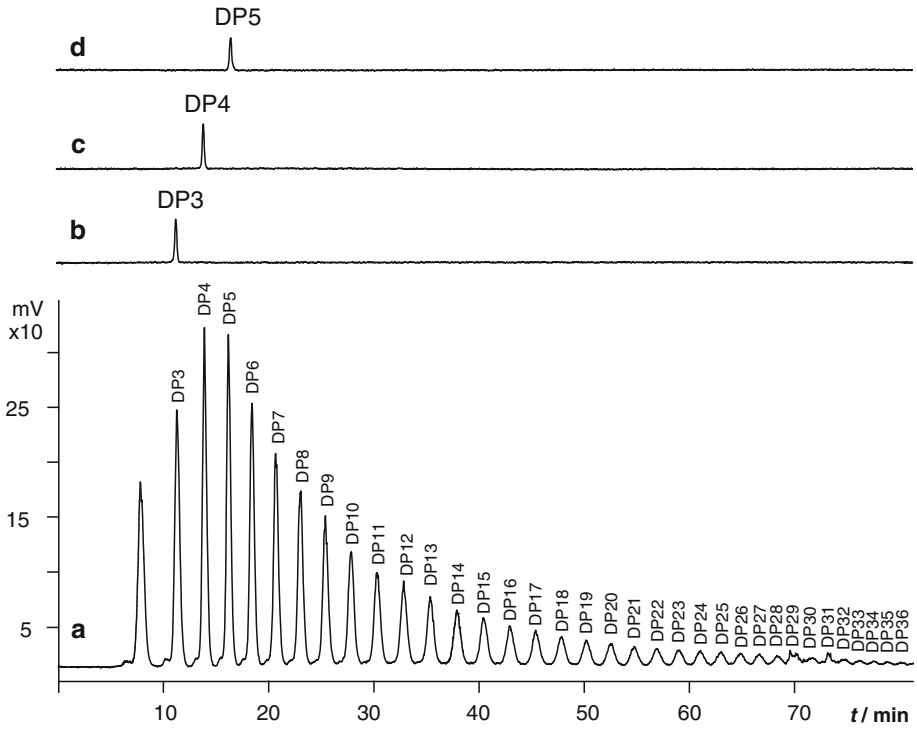

Fig. 3 HPLC chromatogram of the standard inulin sample (**a**), DP3 sample (**b**), DP4 sample (**c**), and DP5 sample (**d**). The gradient program was the following (eluent A: 0.04% aqueous NH_3 solution, B: acetonitrile): 70% B (0 min), 60% B (20 min), 40% B (110 min), 0% B (120 min), 0% B (130 min), 70% B (140 min), 70% B (150 min)

with mass spectrometric detection using atmospheric pressure chemical ionization (APCI). The ion chromatogram was detected in positive detection mode (shown in Fig. 4a). The ion chromatogram shows similar signal characteristics and dispersion to that of ELS detection. The signals were scanned by mass spectrometric detector and the obtained mass spectra are shown in Fig. 4b–j. The molar masses of the components were detected as ammonia adducts and the molecular peaks displayed the highest intensities for DP3 (Fig. 4b) and DP4 (Fig. 4c). Significant molecular peak intensity decreasing tendency has been observed from DP5 (Fig. 4d) to DP7 (Fig. 4f). The molecular peak disappears from the mass spectra of oligomers with higher molecular weights starting at DP8 (Fig. 4g). However, intense fragmentation has been observed in the mass spectrum for the oligomer components, lower molecular weight oligomer fragments have appeared in the spectra, furthermore the mass spectra of DP8–DP11 (Fig. 4g–j) showed only DP5–DP1 fragments, higher molecular weight oligomers (DP12–DP14) showed even poorer fragmentation patterns (spectra not shown).

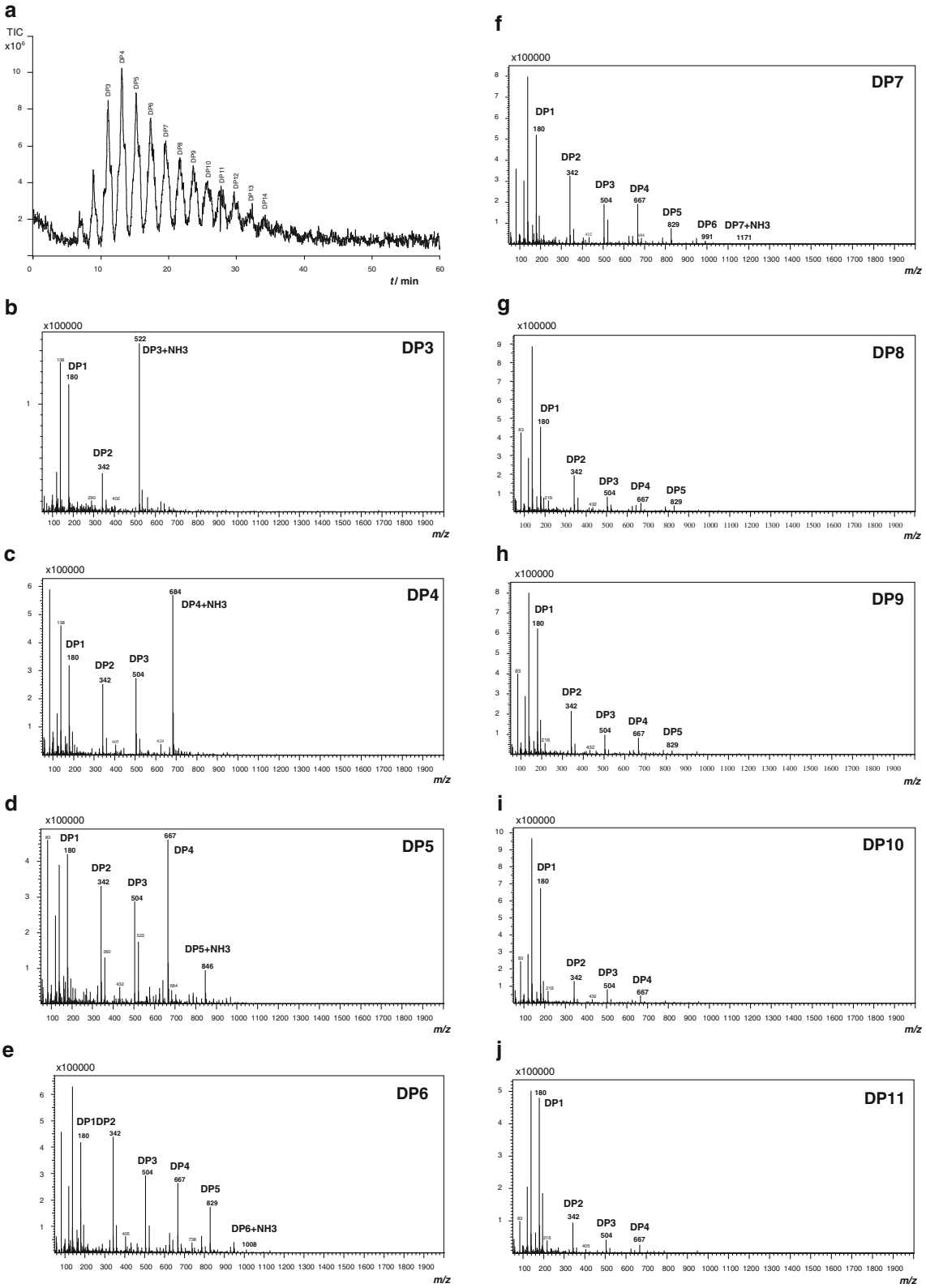

Fig. 4 HPLC–MS ion chromatogram of the standard inulin sample (**a**) and mass spectra of the different components: DP3 (**b**), DP4 (**c**), DP5 (**d**), DP6 (**e**), DP7 (**f**), DP8 (**g**), DP9 (**h**), DP10 (**i**), and DP11 (**j**)

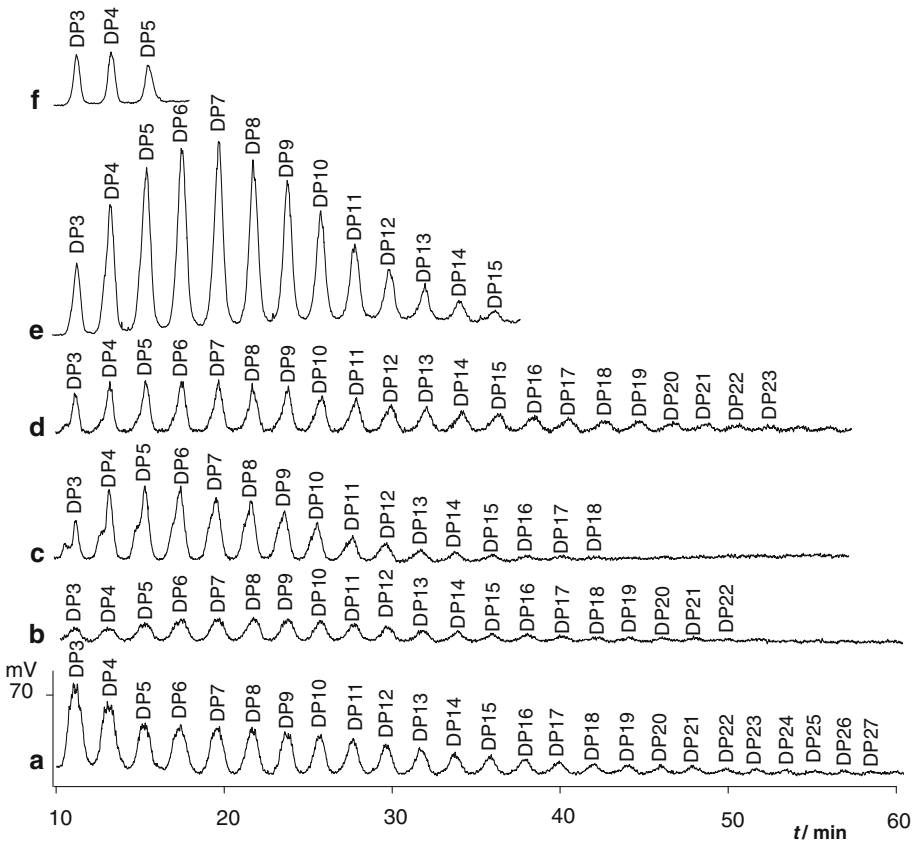

Fig. 5 HPLC chromatograms of different industrial inulin samples, **a** inulin containing food supplement powder, **b** inulin containing soft-drink, **c** industrial inulin powder—I, **d** industrial inulin powder—II, **e** bulk inulin powder, **f** standard solution of DP3, DP4, and DP5 with 1 mg/cm^3 concentration

Several industrial inulin samples have also been examined (ELS detection) and the same signal dispersion has been found. The inulin containing powdered food supplement product (Sia, Riga, Latvia; Fig. 5a) comprised detectable oligomers between DP3 and DP27 having the DP3 signal as the highest one in the chromatogram. Quantitative analysis of this dry food additive powder has been carried out with the result exhibiting 44.8% total inulin content. An inulin containing soft-drink product called ZEN prebiotic soft drink (Buszesz, Hungary; Fig. 5b) had oligomers detected between DP3 and DP22 with total inulin content of 853.0 mg/100 cm^3. Industrial inulin samples (Fructafit-Clr in Fig. 5c and Fructafit-HD in Fig. 5d) contained oligomers with shorter chain length (max. DP18 in case of Fructafit-Clr and max. DP23 in case of Fructafit-HD) and with inulin content around 57% calculated for the dry material. The third sample (Coscura inulin in Fig. 5e) had the shortest chain distribution including DP3–DP15 oligomers, however the largest signal areas (integrals) were established in case of this sample as around 91.0% inulin content was calculated for the dry material.

Subsequent to accomplishment of thermal treatment of the inulin samples at two distinctive temperatures (at 180 and 210 °C) for 10 min, compositions have been investigated by HPLC analysis (results are shown in Fig. 6). Intense chain degradation has been established at both temperatures. At 180 °C (Fig. 6b) higher molecular weight oligomers starting at DP18 disappeared from the reaction mixture, while the intensity of signals between DP3 and DP7 has significantly been increased. Progress of the degradation has been justified when the temperature was increased to 210 °C (Fig. 6c). In this case four major components have been detected between DP3 and DP6, while the presence of higher molecular weight oligomers has been found to be less pronounced.

Conclusions

A reliable HPLC method has been applied for the analysis of inulin-type fructans using evaporative light scattering and mass spectrometric detection techniques, the distribution of different oligomers in the sample has been determined. The mass spectrometric detection allowed the identification of several oligomer components through intense fragmentation in lower molecular weight components. Several industrial food samples have been investigated and the distribution of different oligomers in real samples has been determined. The thermal stability has also been investigated and HPLC experiments have demonstrated that significant chain degradation took place. One

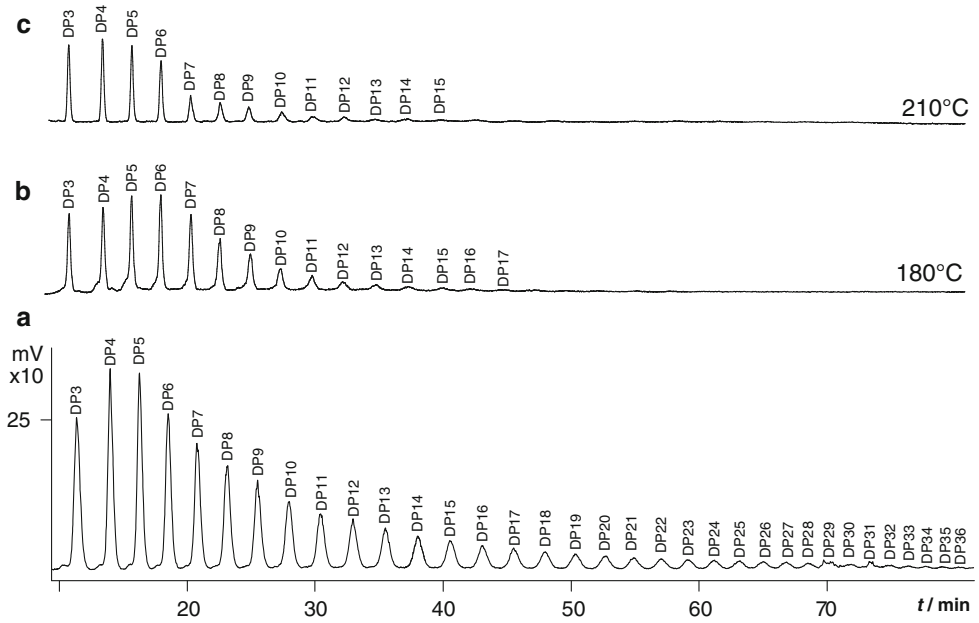

Fig. 6 HPLC chromatograms of thermally treated inulins compared to the standard inulin sample (**a**), 180 °C (**b**), and 210 °C (**c**)

of the most important conclusions of the experiments is that fructans with or without thermal treatment may be directly analyzed with the application of a simple sample preparation procedure and without previous enzymatic or chemical hydrolysis avoiding the usage of complex digestion and extraction processes.

Experimental

Chicory and artichoke inulins for chemical use were purchased from Sigma-Aldrich (Steinheim, Germany), Fluka (Buchs, Switzerland), Sensus (Roosendaal, The Netherlands), Beneo Orafti (Belgium), Ökoszervíz Kft. (Szentendre, Hungary), Sia (Riga, Latvia), and from Buszesz (Hungary). Chemical reagents applied for chromatography were obtained from Sigma-Aldrich (Steinheim, Germany). High purity 1-kestose, 1,1-kestotetraose, and 1,1,1-kestopentaose (Fig. 2) were used as standards for the identification of low molecular weight oligomers and have been obtained from Sigma-Aldrich (Steinheim, Germany). The sample preparation procedure was carried out without previous enzymatic or chemical hydrolysis of the samples. The chromatographic detection was carried out by using ultra-high-purity water (0.04% NH_3) and acetonitrile as gradient mobile phases. Liquid chromatography was carried out using a HPLC system including a SIL-20A autosampler, an LC-20AB binary pump, a DGU-$20A_3$ degasser, a CBM20A communication module, a CTO-20A column chamber from Shimadzu Manufacturing Inc., and a Prevail Carbohydrate column (250 × 4.6 mm, 5 μm) from Altech Association Inc. (Deerfield, Ireland). Sample preparation resumed only to dissolving samples in water and filtering. Hot water (85 °C, 10 cm^3) was added to the inulin samples (1 g), the solutions were sonicated and filtered (0.45 μm, membrane) into a glass autosampler vial for injection, standard solutions ($\sim$1 mg/cm^3) were prepared using low molecular weight oligomers. Detection and identification of inulin oligomers were performed using a PL-ELS-2100 evaporative light scattering liquid chromatographic detector. Injection volume was 10 mm^3 and the flow rate of the eluent was 0.4 cm^3/min with a gradient mobile phase of acetonitrile-0.04% NH_3 in water. The structural identification of different oligomer components has been carried out by APCI ionized mass spectrometer detection in positive ion mode, the 50–2,000 *m/z* region was scanned and 1.5 kV detector voltage was used. The interface temperature was 250 °C, nebulizing gas flow 0.18 dm^3/min, and the heat block temperature was 200 °C.

Thermal stability of the inulin standard has also been investigated by being treated at 180 and 210 °C for 10 min, and subsequently HPLC–ELS examinations have been performed.

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